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(57) Abstract

A cellular immune response is induced to a non–immunogenic or weakly immunogenic target protein expressed by tumor cells, by administering to a mammalian subject an amount of a therapeutic antigen effective to induce a cellular immune response to the target protein. The therapeutic antigen is an immunogenic peptide having an MHC–binding portion which binds to the major histocompatibility complex (MHC) and an immune–recognition portion which is recognized by T–cells. The therapeutic antigen is derived from the non–immunogenic or weakly immunogenic target protein such that the MHC–binding portion binds to MHC with a greater affinity than the target protein without material alteration of the immune–recognition portion. The therapeutic antigen may include a sorting signal which directs the transport of the therapeutic antigen into the endoplasmic reticulum and into the endosome/lysosome to facilitate loading of the peptide onto MHC class I and class II molecules, respectively, to facilitate loading of the protein onto MHC molecules for presentation to the immune system.

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VACCINATION STRATEGY TO PREVENT AND TREAT CANCERS

BACKGROUND OF THE INVENTION

This application relates to a vaccination strategy for the prevention and treatment of cancer in animals, including humans, and to compositions useful in implementing this strategy. The invention is particularly applicable to the prevention and treatment of cancers where the pathogen or cancer is inherently a poor immunogen which does not stimulate an effective immune response.

Cytotoxic T-lymphocytes (CTLs) can play a central role in the rejecting tumors. Tumor antigens recognized by CTLs generally originate from one of three sources: (1) viruses; (2) self proteins expressed during development or differentiation (including proteins sometimes referred to as "differentiation antigens") and (3) mutant or aberrantly expressed proteins. CTL responses against antigens of these three types present different problems, requiring different solutions.

In the case of innumogenic antigens of viral origin, Lipford et al. *Immunology* 84: 298-303 (1995) discloses the use of peptide engineering to develop modified antigens based on human papilloma virus tumour antigen E6 (HPV E6). These modified antigens are derived from the wild-type structure of HPV E6 via the introduction of mutations which increase the stability of the antigen's interaction with the major histocompatability complex (MHC). Lipford suggests that these antigens will be effective in promoting a CTL response, although no *in vivo* data is reported in which such a response is actually demonstrated.

In the case of mutant or aberrantly expressed genes, US Patent No. 5,662,907 to Kubo et al. discloses nine residue antigenic peptides based directly on the tumor-associated gene MAGE-3 which are shown to be effective to induce CTL responses. This patent further suggests that modifications to these peptides to increase the stability of interaction with the MHC may be desirable.

Neither Lipford et. al. nor Kubo et al. address the more difficult challenge of constructing an immunotherapeutic agent to promote an immune response against non-immunogenic or weakly immunogenic proteins, including some viral protein and many self-proteins. Such self-proteins are expressed during development or differentiation, and may be

present on both normal and tumor tissues, albeit to different extent. For this reason, they are not recognized by the immune system, and antigens derived directly from such antigens are not effective to induce an immune response even when administered with an adjuvant system. Furthermore, Roberts et al, *Current Opinion in Immunology* 8:628-636 (1996) have noted that "the T cell epitopes derived from the normal melanocyte proteins ... appear to bind to HLA-A2.1 with low affinities relative to foreign and viral epitopes. Peptides that are derived from the gene products and that bind to MHC gene products with high affinity may be expressed at high density on the surface of melanocytes and may result in the induction of tolerance." Nevertheless, the ability to induce a CTL response, without tolerance, to such antigens would be of substantial benefit in the treatment of a variety of cancers.

It is an object of the present invention to provide such a method and compositions for use in the method.

SUMMARY OF THE INVENTION

In accordance with the present invention, a cellular immune response is induced to a non-immunogenic or weakly immunogenic target protein expressed by tumor cells by administering to a mammalian subject an amount of a therapeutic antigen effective to induce a cellular immune response to the target protein. The therapeutic antigen is an immunogenic peptide having an MHC-binding portion which binds to the major histocompatability complex (MHC) and an immune-recognition portion which is recognized by T-cells. The therapeutic antigen is derived from the non-immunogenic or weakly immunogenic target protein such that the MHC-binding portion binds to a class I or class 2 MHC molecule with a greater affinity than the target protein without material alteration of the immune-recognition portion. In addition, the therapeutic antigen may include a sorting signal which directs the transport of the therapeutic antigen into the endoplasmic reticulum and into the endosome/lysosome to facilitate loading of the peptide onto MHC class I and class II molecules, respectively, to facilitate loading of the protein onto MHC molecules for presentation to the immune system.

BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1A and B show the efficacy of CTL priming by SSI and SEI peptides;

Fig. 2 shows the antigenicity and immunogenicity for SSI and SEI peptides;

Figs. 3A-3G show the *in vivo* ability of SSI peptide to protect mice against transplantable tumor expressing SEI;

Figs. 4A-4C show the ability of vaccination with heteroclitic immunogen to eradicate 3 day tumors in mice;

Fig. 5 shows the results of an RMA-S stabilization assay (Kb-binding assay) of the natural melanoma gp75 peptide TWH and its engineered heteroclitic variant TAY for binding with k^b;

Figs. 6A and 6B show results of studies on the TAY peptide as a heteroclitic immunogen for native gp75 melanoma peptide, TWH; and

Figs. 7A and 7B shows the *in vivo* efficiacy of a heteroclitic melanoma vaccine.

DETAILED DESCRIPTION OF THE INVENTION

In tumor transplantation models in mice, cytotoxic T lymphocytes (CTL) are typically the primary effector cells. CTL recognize MHC class I-associated peptides expressed by tumors, leading to tumor rejection. Peptides presented by cancer cells can originate from viral proteins, altered proteins derived from genetic alterations, or normal self proteins regulated during differentiation. In the latter case and in the case of some viral proteins, the immune system is tolerant of or otherwise fails to recognize the antigens, such that they are inherently non-immunogenic or only weakly immunogenic in the host, and are unable to induce activation and differentiation of effector CTLs. Such antigens therefore are of at most limited therapeutic utility in conventional approaches to immunotherapy.

The present invention is directed to a method and composition for inducing a cytotoxic T lymphocyte (CTL) immune response to non-immunogenic or weakly immunogenic self-proteins expressed on tumor cells. CTLs recognize target antigens in the form of short, intracellularly-processed peptides, presented by self major histocompatibility complex (MHC) encoded class I molecules (pep:class I). Upon binding of the antigen-specific T cell receptors (TCR) on a CTL to its cognate peptide/MHC complex on a target tumor cell, the target cell is lysed, and the tumor reduced or eliminated. To develop into effector CTLs capable of cell lysis, naive precursor CTLs (pCTLs) have to be activated. This pCTL activation requires two signals: the first, or stimulatory signal (signal 1), transmitted via the TCR/CD3 complex, and the second, or costimulatory signal (signal 2), delivered by

professional antigen presenting cells (2-4). In the thymus, a strong signal 1 will induce negative selection of immature thymocytes, regardless of signal 2 (5,6). In the periphery, the same strong signal 1 will induce immunity (including pCTL to CTL differentiation) or anergy, depending on the presence or absence of signal 2 (2-4). By contrast, even a weak signal 1, without signal 2, can be sufficient for target cell lysis by differentiated CTLs (7). This means that a whole class of antigenic peptides exists that, although poorly immunogenic (i.e. unable to induce CTL immunity), can theoretically serve as molecular targets for lysis by differentiated effector CTLs which recognize such antigens. Such antigenic, but poorly immunogenic, peptides remain invisible to the naive pCTL.

Self-antigens, including for example differentiation antigens, which are expressed by both normal and tumor cells to differing degrees are generally of this type. The self-antigens are inherently non-immunogenic or weakly immunogenic in the host organism, and no CTL response is developed. The present invention exploits the presence of such poorly- or non-immunogenic peptides at the surface of tumor cells by designing immunogenic variants of these peptides that prime a CTL response that crossreacts to the original targeting peptide. This strategy is attractive, because the relative invisibility of poorly immunogenic self peptides to the immune system is actually advantageous. Specifically, unlike self peptides that provide strong signal 1 (8), poorly immunogenic peptides fail to induce tolerance, and the T cell repertoire reactive against them is intact and available for activation with immunogenic peptide variants.

According to the nomenclature used for variants of a pigeon cytochrome C peptide (9), peptides of higher biological potency than the original peptide were called heteroclitic. Crystal structure analysis revealed that, of the 8-10 amino acid residues of a MHC class I-bound peptide, roughly half point into the solvent, and can interact directly with the TCR via their side chains (10-12). The other half are buried by class I and are not directly accessible to the TCR (10-12). Heterocliticity has been achieved by substituting amino acids that contact MHC class I, the TCR, or both (13,14).

The present invention provides heteroclitic peptides derived from non-immunogenic or weakly immunogenic self-proteins that are expressed by tumor cells. As used herein, the term "derived from" refers to peptides which have an amino acid sequence which is based at least in part on the structure of naturally occurring non-immunogenic or weakly immunogenic

self-proteins. Preferred naturally-occurring proteins from which the heteroclitic peptides of the invention are derived are those which are expressed on the surface of tumor cells (in some cases at greater levels when compared to normal, non-tumor cells) and which are treated by the immune system as self-proteins such that they do not themselves induce a CTL immune response even when administered with an adjuvant. Most preferred candidates for use as the starting peptide in the present invention are self peptide that, relative to an optimal binding peptide (e.g. OVA-8 or SIINFEKL [Seq. ID No. 3], in the case of H-2K^b or influenza matrix peptide 58-66 of GILGFVFTL [Seq. ID No. 4] in the case of HLA-A0201) exhibit 25% or less binding at a peptide concentration of 10 μM, and 10% or less binding at 1 μM. The binding affinities can be determined with essentially any assay, including the RMA-S MHC Class I stabilization assay described in the Examples.

Preferred peptides which can be used as the basis for heteroclitic peptide vaccines in accordance with the invention are human peptides which bind, albeit weakly, to the HLA-A* 0201. Non-limiting of such natural, human peptides include:

Telomerase reverse transcriptase (TERT) peptide DVLVHLLAR [Seq. ID No. 5];
CD20 peptides RMSSLVGPT [Seq. ID No. 6] and RMSSLVGPV [Seq. ID No. 7];
Prostate's PSMA LLQERGVAYI [Seq. ID No. 8].

Principles of peptide binding to MHC class I and class II molecules are sufficiently similar, and the knowledge about the peptide motifs (Rammensee et al. *Immunogenetics* 41:178-228, 1995 and ref. 13) and orientation of the peptide side chains when bound to MHC class II available (ref. 10), for one skilled in the art to apply the above findings to the MHC class II peptides for activation of CD4 T cells as well. Thus, the heteroclitic peptide in accordance with the invention can also be one in which the MHC-binding domain binds to enhanced affinity to an MHC Class II molecule on an antigen presenting cell, and the immune-recognition domain binds to the TCR of a CD4+ T cell. Identification of target peptides, which will generally be 9 to 14 amino acids in length, is done in substantially the same manner as for target peptides which bind to MHC-Class I molecules. Such screening is described in Rammensee et al., *supra* and Wang et al., *Science* 284: 1351-4, 1999 Exemplary target proteins in this case include, but are not limited to the antigens mentioned for MHC class I, as well as human CDC27, BCR-ABL translocation product frequently found in B cell

lymphoma, and mutated or normal p21ras (See, Wang et al., *supra*; Cheever. M.A. et al., *Annals NY Acad. Sci.* 690: 101-112, 1993)

The heteroclitic peptides of the invention differ from these natural forms by the incorporation of mutations into the peptide to increase the binding affinity of the major histocompatability complexd (MHC) for the peptide, without altering in any material way the portion of the peptide which is presented by the MHC for immune recognition. In this way, the modified peptide can induce an immune response which is effective against the naturally occurring epitope. MHC binding motifs and the knowledge of class I: peptide:TCR structure are used to design heteroclitic CTL vaccines according to the invention that exploit the expression of poorly immunogenic tumor peptides. The cellular immune response is naive with respect to these modified antigens, such that tolerance has not developed. The CTL response that develops, however, is capable of recognizing the original antigen, and is thus an effective therapeutic agent to induce an immune response against cells, including tumor cells, expressing the original antigen. The identification of peptides for use in the present invention can be approached using various strategies, including elution of MHC-bound peptides and protein scanning.

In general, once a suitable non-immunogenic or weakly immunogenic peptide has been identified, the heteroclitic peptide is created by substituting amino acids within the portion of the peptide which will bind to the MHC. Amino acid substitutions are generally considered as being either conservative or non-conservative, depending on whether the nature (size, hydrophobicity, hydrophilicity, etc) of the starting amino acid and the replacement are similar or different. For purposes of converting a non- or weakly immunogenic peptide into a heteroclitic peptide in accordance with the invention, one or more non-conservative substitutions are most likely to be effective. Thus, for example, an amino acid such as Trp, with a large bulky side chain, may be replaced with an amino acid such as Ala, with a small side chain; or an amino acid such as Glu, with a hydrophilic and negatively charged side chain, may be replaced with an amino acid such as Ile, with a hydrophobic uncharged side chain; or a charge reversal from a positively charged side chain, such as Lys or Arg, to a negative charge, such as Glu. To design peptides that are heteroclitic for polyclonal CTL responses, one should optimize pep:class I binding, since this property correlates with immunogenicity (7,15). At the same time, the peptide:TCR contact should not be disturbed,

to maximize the potential crossreactivity between the heteroclitic and the original, non-immunogenic targeting peptide.

The peptide of the invention may be further modified by coupling the peptide with a sorting signal which directs the transport of the peptide into the endoplasmic reticulum to facilitate loading of the protein onto MHC molecules for presentation to the immune system. Suitable sorting signals for directing intracellular transport of the expressed antigen to the endoplasmic reticulum (or retention therein) include the signal region

Pro Ser Arg Asp Arg Ser Arg His Asp Lys Ile His SEQ ID No. 1

which has been shown to retain a viral glycoprotein in the endoplasmic reticulum. Rose et al. "Altered cytoplasmic domains affect intracellular transport of the vesicular stomatitis virus glycoprotein" *Cell* 34: 513 (1993); Bartido et al. "Processing of a viral glycoprotein in the endoplasmic reticulum for class II presentation" *Euro. J. Immunol.* 25: 22111-2219 (1995). See also, commonly assigned International Patent Publication No. 98/04720, which is incorporated herein by references. Another sorting sequence which can be used has the sequence

MRYMILGLLALAAVCSA SEQ. ID No. 2,

J. Exp. Med. 174:489-492 (1991), as described in the examples below. Alternatively, in the case of MHC class II-binding peptides, one may use the known endosomal sorting signals from the invariant chain-DDQRDLISNNEQLPMLG [Seq. ID No. 22] *Curr. Opin. Immunol.* 6:57-63 (1994) or the melanosomal protein sorting signal EANQPLLTD [Seq. ID No. 23] *J. Biol. Chem.* 130:807-820 (1995).

The heteroclitic peptides of the invention may be chemically synthesized, or they may be synthesized by expression from appropriate oligonucleotide sequences either *in vitro* or by *in situ* synthesis *in vivo*.

Vaccination with the compositions of the invention may be performed by using peptides, or by genetic immunization using DNA encoding the peptide. The vaccine is administered in an amount effective to promote an immune response, and preferably a CTL

response, to the naturally occurring epitope of the tumor cell. The determination of appropriate levels will require testing on individual vaccines, and individual species of animal (including man) to be treated. Such testing is a matter of routine optimization, however, and lies within the skill in the art.

The *in vivo* potency of the vaccination strategy of the invention was demonstrated using self differentiation antigens as models. A peptide from a relevant self antigen of the tyrosinase family expressed by melanoma cells (gp75) was used to design a heteroclitic peptide vaccine that successfully induced tumor protection. The results establish the *in vivo* applicability of heteroclitic immunization against tumors, including immunity to poorly immunogenic self proteins. The heteroclitic immunogens elicit antitumor CTL responses that crossreact to the original poorly immunogenic antigens. Therefore, to design peptides that are heteroclitic for polyclonal CTL responses, one should optimize pep:class I binding, since this property correlates with immunogenicity (7,15). At the same time, the peptide:TCR contact should not be disturbed, to maximize the potential crossreactivity between the heteroclitic and the original, non-immunogenic targeting peptide. We sought to test whether this strategy could induce antitumor CTLs reactive to non-immunogenic targeting peptides. We demonstrate successful *in vivo* induction of crossreactive CTLs in two tumor models, using an engineered viral peptide variant expressed as a tumor antigen (a model of a tumor antigen of viral origin) and a self antigen expressed in melanomas (a model of non-mutated, differentiation antigen). Induced CTLs were biologically active in vivo, and were able to effect rejection of both newly implanted and established day 3 tumors.

EXAMPLES

MATERIALS AND METHODS

Mice. Female C57BL/6 (B6) mice were purchased from the National Cancer Institute breeding program (Frederick, MD). B6.C-H-2^{bm8} (bm8) mice were bred in the MSKCC vivarium, from a breeding stock obtained from the Jackson Laboratory, (Bar Harbor, ME) via Dr J. Sprent (The Scripps Research Institute, La Jolla, CA). All mice entered the study between 7 and 10 weeks of age.

Antibodies, in vivo CD8 depletion, flow cytometry and class I stabilization assays. The anti-CD8 mAb, 53.6.7 (rat IgG) and the anti-K^b mAb Y3, (mouse IgG_{2b}), both obtained

from ATCC, were produced as ascitic fluid in our lab. For *in vivo* CD8 depletion, 100 µl of ascitic fluid was injected i.p. on days -7 and -3 relative to tumor challenge, which was denoted as day 0. Phycoerythrin (PE) -conjugated anti-mouse IgG_{2b} was purchased from Fisher Biotech (Malvern, PA). Flow cytometry and the class I stabilization assays were performed exactly as described (16) using a FACScan instrument equipped with Lysys II software (Becton Dickinson, Mountain View, CA).

Construction of minigenes. Inserts coding for the ER insertion sequence (17,18) [Seq. ID No. 2] followed by the peptides SEI (SEIEFARL [Seq. ID No. 9]) and SSI (SSIEFARL [Seq. ID No. 10]), based upon the immunodominant sequence 498-505 of the *Herpes simplex* virus glycoprotein B, or peptides TWH (TWHRYHLL [Seq. ID No. 11]) or TAY (TAYRYHLL [Seq. ID No. 12]), based upon the sequence 222-229 of the melanoma gp75 protein, were produced by multistep PCR. All oligonucleotides were purchased from Retrogen (San Diego, CA). For the construction of pERIS-SSI and pERIS-SEI minigenes, the PCR reactions were done with two common oligomers, C1 (GGG AAG CTT ACC ATG AGA TAC ATG ATC CTG GGC CTG CTG [Seq. ID No. 13]), C2 (GGC CTG CTG GCC CTG GCC GCC GTG TGC AGC GCT GCC AGC [Seq. ID No. 14]) and the specific oligomers SSI (TTT CTC GAG TCA CAG CCT GGC GAA CTC GAT GCT GCT GGC AGC [Seq. ID No. 15]) or SEI (TTT CTC GAG TCA CAG CCT GGC GAA CTC GAT CGA GCT GGC AGC [Seq. ID No. 16]). C2 and SSI or SEI were first joined in 50 µl reactions consisting of 300µM dNTP's and 20µg/ml of primers, for 30 cycles at 95°C for 30 s, 30°C for 60 s and 72°C for 30 s. 5 µl of the product was added to 45 µl of C1 and either SSI or SEI, at the same primer and dNTPs concentrations and another PCR performed for 10 cycles as above, followed by 30 cycles at 95°C for 1 min and 72°C for 2 min. For the construction of pERIS-TAY and pERIS-TWH, the common oligos C1.1 (GGG AAG CTT ACC ATG AGA TAC ATG ATC CTG GGC CTG CTG GCC CTG GCC GC [Seq. ID No. 17]) and C2.1 (GGC CTG CTG GCC CTG GCC GCC GTG TGC AGC GCT GCT [Seq. ID No. 18]) were used with the specific oligos TAY (TTT CTC GAG TCA CAG CAG GTG GTA TCT GTA GGC GGT GGC AGC GCT [Seq. ID No. 19]) or TWH (TTT CTC GAG TCA CAG CAG GTG GTA TCT GTG CCA GGT GGT AGC GCT [Seq. ID No. 20]). The first step was exactly as described above. However, the second step was done for 40 cycles at 94°C, for 20 s, followed by 60°C for 20 s and 72°C for 45s. Thus engineered products

contain the Hind III and Xho I sites, which were used for cloning into a LacZ-containing pCR2 cloning vector (Invitrogen, San Diego, Ca). Clones that scored positive by blue/white screening were digested by Hind III and Xho I, and the inserts recloned into pCDNA3 to obtain the appropriate expression constructs, pcERIS-SSI, pcERIS-SEI, pcERIS-TWH and pcERIS-TAY. The transfer was confirmed by sequencing.

Cell Transfection. 10μg of linearized plasmid DNA was electroporated into 10 million RMA-S cells in 500 μl of Optimem (Gibco) containing 5% FCS, using a Gene Pulser (Biorad, Hercules, CA) set at 220 V and 960 μF. 48 hours later, the cells were incubated in the presence of 600μg/ml G418 (Gibco-BRL). G418-resistant clones were selected from the 96-well plates with less than 30% positive wells.

Peptide and gene gun immunization, in vitro CTL restimulation and CTL assays. The five peptides used in this study, HIV-10 (RGPGRAFVTI [Seq. ID No. 21]), SSI (SSIEFARL [Seq. ID No. 10]), SEI (SEIEFARL [Seq. ID No. 9]), TWH (TWHRYHLL [Seq. ID No. 11]), and TAY (TAYRYHLL [Seq. ID No. 12]) were synthesized by the MSKCC Microchemistry Core Facility, and were HPLC purified to >98% purity. Peptide immunization using the synthetic immune adjuvant TiterMax (CytRx Inc., Norcross, GA), referred to as pep/TM in the text, CTL restimulation and ^{51}Cr-release assays were performed as previously described (19). Briefly, mice were immunized in the footpad with 10μl of the pep/TM emulsion (mixed according to the manufacturer's instruction) containing 5μg of the indicated peptide. Seven days later, spleen cells from the immunized mice were restimulated in vitro with syngeneic, irradiated (30 Gy), peptide-coated (1μg/ml, 2 ml/spleen, 1 h at 37°C following by three washes) cells. Five days later, cytolytic activity was assessed in a standard ^{51}Cr-release assay. Genetic immunization using DNA-coated gold particles was performed exactly as described using a gene gun generously provided by Powderject, Inc., Middleton, WI (20). 100μg of DNA from the plasmids described in the previous section was mixed with 0.95-2.6 μm diameter gold particles, in the presence of 0.05-0.1 μM spermidine. $CaCl_2$ (1.5 mM) was added in a dropwise fashion to this mixture while vortexing. After precipitation, the gold-plasmid DNA complex was washed three times in 100% ethanol, and 7 ml of ethanol added to achieve a bead-loading rate of 0.5 mg of gold to 1.0 μg plasmid DNA/injection. This solution was then instilled into plastic Tefzel tubing, the ethanol gently drawn off, and the tube purged under nitrogen gas at 400 ml/min for drying. The tube was then cut into 0.5 inch

bullets. The gold particles in the "bullets" were injected into the skin of anesthetized mice using a helium-driven gene gun (Powderject, Inc.). The skin was shaved and depilated prior to injection (20). Four injections at 400 pounds/square inch (p.s.i.) were delivered to each mouse, one to each of the abdominal quadrants, for a total of 4μg of plasmid DNA/mouse. Seven days later, spleen cells were restimulated and CTL activity determined as described for pep/TM.

Tumor challenge and follow up. 5 x 10^5 RS-H2E or RS-Null cells or 1 x 10^5 B16F10LM3 (designated B16 in the text) melanoma cells (derived from B16F10 melanoma cells, a gift of Isaiah Fidler, MD Anderson Cancer Center, Houston TX) were injected into the shaved left flank of the mice. Mice were then monitored three times a week for tumor growth, initially by palpation and subsequently, when tumor growth was manifest, using Vernier calipers. Measurements were achieved by obtaining the maximum diameter of the tumor and the diameter perpendicular to the maximum, that were then multiplied and the product of these two values reported as tumor size. Tumor growth curves are shown for individual mice/experiment. Mice surviving tumor challenge were followed for a minimum of 60-90 days. The mice were euthanized if the maximum tumor diameter exceeded 10 mm, or if the tumor became ulcerated.

Example 1

Heteroclitic vaccination in an engineered lymphoma model

To establish the principle of heteroclitic immunization against tumors, we used an engineered peptide (SSI) based on the sequence of the Herpes simplex virus glycoprotein $B_{498-505}$ peptide (SEI), which also served as a model of a tumor antigen of viral origin. The SEI peptide (sequence SEIEFARL [Seq. ID No. 9]), binds poorly to the murine MHC I molecule H-2K^b (K^b) owing to the electrostatic repulsion between the negatively charged glutamic acid residues at the buried position 2 of the peptide (P2E) and the adjacent position 24 (MHC24E) of K^b (16). To evaluate CTL responses to peptide priming of B6 mice, three mice per group were vaccinated with indicated peptides in adjuvant (pep/TM). Seven days later, spleen cells were restimulated *in vitro* and CTL responses of individual mice tested in a ^{51}Cr release assay using K^b-expressing EL-4 target cells pulsed with 10 μM of the immunizing peptide, as described (19). The lysis of unpulsed EL-4 cells (always <10%) was subtracted, and results

shown for individual mice at indicated effector:target ratios. The results, which are shown in Fig. 1A, are representative of over 45 mice tested in at least 10 independent experiments. Indistinguishable results were obtained using DNA immunization by particle bombardment (Table 1). Owing to poor binding, the SEI peptide cannot elicit a CTL response in K^b-bearing C57BL/6 (B6) mice following immunization with peptide/adjuvant (Fig. 1A, circles, and Table 1) or with genetic immunization using DNA delivered by particle bombardment (Table 1).

As shown in Fig. 1B, peptide immunogenicity correlates to peptide binding. bm8 mice respond to peptide priming by SSI (squares) and SEI (circles), both of which bind well to K^{bm8} (16). Results are representative of at least 25 mice/strain tested in at least six independent experiments. Methods and data representation were as described in A. However, SEI was a good immunogen in B6.C-H-2.^{bm8} (bm8) mice (Fig. 1B, circles), that express a natural K^b variant, K^{bm8}. This class I molecule has an E24→S mutation that enables strong SEI binding (16). These results demonstrated that the immunogenicity of the above peptide correlated directly to peptide binding, and that the absence of a response in B6 was not due to deficient vaccine formulation.

The natural viral peptide from which H2E was derived, SSI (SSIEFARL [Seq. ID No. 10], also referred to as HSV-8), differs from SEI by having a serine (P2S) instead of the glutamic acid (P2E) in position 2. SSI would be predicted to remove the electrostatic repulsion between the peptide and K^b. Indeed, SSI bound a hundred fold better than SEI to K^b (16) and was strongly immunogenic for B6 CTLs (Fig. 1A, squares, and Table 1). We next asked whether SSI-induced CTLs could lyse cells bearing the SEI peptide in a crossreactive fashion, and found that this was the case when target cells were coated with high concentrations of SEI (> 10 μM) *in vitro*.

While these *in vitro* results were encouraging, their *in vivo* relevance for tumor immunity was obscure. Particularly, it was unclear whether intracellularly expressed weak MHC binders, such as SEI, would be processed and presented efficiently enough for target cell lysis. To address that issue, we expressed SEI in a B cell lymphoma, RMA-S (21). RMA-S has a chemically induced deletion of one of its transporter associated with peptide processing (TAP) genes, *Tap-2*. This deletion prevents the vast majority of cytosolically processed peptides from entering the ER and binding to empty class I molecules, which leads

to decreased expression of stable class I molecules at the surface of RMA-S cells. The TAP defect was circumvented using a minigene encoding an endoplasmic reticulum insertion sequence (ERIS) (17) followed by the SEI peptide. Fusion proteins encoded by such ERIS-containing minigenes were previously shown to insert the attached class I binding peptides into the ER, thereby bypassing the TAP defect and partially restoring the surface expression of pep:class I (17,18). Indeed, the experimental tumor line, RS-SEI (RMA-S cells transfected with the pERIS-SEI plasmid), had a higher surface level of K^b than RS-Null cells (transfected with the "empty" control plasmid, pcDNA3), as measured by flow cytometry. The mean relative K^b fluorescence intensity for RS-SEI was 123 as compared to only 79 for RS-null. These observations were consistent with the efficient ERIS-mediated import of SEI into the ER.

Three anti-SSI CTL lines (derived from individual B6 mice by peptide immunization) were tested for the ability to lyse K^b expressing target cells in a standard ^{51}Cr-release assay. The results are summarized in Fig. 2, where the closed squares are data for target cells expressing RS-SEI and the open squares are data for target cells expressing RS-Null. As shown, RS-SEI was lysed by anti-SSI CTL line, while RS-Null was not. Six more lines were tested and gave identical results. These tests demonstrated that CTLs induced by the heteroclitic vaccine crossreacted on the SEI:K^b expressed by the minigene-transfected cells.

To test the potency of SSI as a heteroclitic vaccine *in vivo*, mice were immunized with either the heteroclitic (SSI) or the parental peptide (SEI) and challenged with RS-SEI or RS-Null tumor lines. Ten B6 mice per group were vaccinated with peptides SSI, SEI or control PBS (19) emulsified in the synthetic adjuvant TiterMax. Seven days later, animals were challenged with $5x10^5$ RS-SEI or RS-Null cells subcutaneously. Nodules were palpable three days after challenge. Numbers on figures show numbers of tumor-free mice at 90 days. A seventh group also received SSI and was challenged with RS-SEI, but the animals were depleted of $CD8^+$ cells by administration of an anti-CD8 mAb before the challenge. Tumors were measured as described in Methods, and results shown as tumor growth curves. All tumor-free mice remained free of tumors for >90 days. DNA vaccination yielded identical results (Table 2). Tumor growth curves among the different groups of challenged mice for a typical experiment are shown in Figs. 3A-3G. The only group protected was the one

vaccinated with the heteroclitic vaccine (SSI) and challenged with the tumor line expressing SEI. Numbers shown in the inset of each figure shows cumulative tumor survival for all mice within the indicated experiment. These results clearly demonstrate the antigenic specificity of the response, and confirm the *in vitro* findings that SEI cannot induce a protective immune response in B6 mice (Figs. 3A-3G). Heteroclitic protection was dependent on $CD8^+$ cells, because mice depleted of $CD8^+$ cells by antibody treatment were not protected (Figs. 3A-3G, Table 2). Identical results were obtained with genetic immunization (Table 2).

The ability of the heteroclitic immunogen to induce rejection of three day tumors was next investigated, using both peptide (19) and genetic (20) vaccination. B6 mice were injected with the RS-SEI tumor as described above. Three days later, when palpable tumors appeared (2-3 mm in diameter), the mice were injected with DNA constructs indicated in Figs. 4A-4C and tumor growth scored. Successful rejection was achieved by genetic immunization only (Figs. 4A-4C and Table 2), consistent with our previous findings that genetic vaccination may be more potent than peptide vaccination (20). These results establish the *in vivo* relevance of heteroclitic immunization.

Example 2

Heteroclitic vaccination against a tyrosinase-family epitope of a melanoma

We next sought to test the applicability of this approach using naturally occurring tumor antigens. As a model, we selected the *brown* locus product, also known as the tyrosinase-related protein-1, or gp75. This glycoprotein is a lineage-specific self antigen, present in melanocytes and expressed in melanomas (22,23). The product of the *brown* locus is a relevant cancer antigen, recognized by both antibodies and T cells in patients with melanoma (22-24). In mice, passive and active immunization against gp75 results in both melanoma rejection and manifestations of autoimmunity (25,26). However, in the mouse model, CTL immunity against gp75-expressing melanoma cells was not induced by immunization with tumor cells plus adjuvant, tumor cells engineered to express cytokines or with purified gp75 protein (26).

Using the canonical K^b-binding motif (27) to scan the amino acid sequence of gp 75 (28), five potential epitopes were identified. Synthetic peptides corresponding to these epitopes were used in both CTL inhibition and class I stabilization assays to determine their

ability to bind to K^b. Peptide TWH, corresponding to gp75 residues 222 to 229 (TWHRYHLL [Seq. ID No. 11]), exhibited similar binding characteristics to SEI and was selected for further experiments. Since this peptide has two bulky side chains not commonly found at the buried, MHC-contacting positions 2 and 3 (P2W and P3H), we suspected that they might sterically impair pep:K^b binding. Based on the available sequence/motif information for K^b-binding peptides, and on the crystallographic data on the pep:K^b structures (10-12,27), we changed these two residues to A and Y, respectively. The variant designed in this manner, named TAY (TAYRYHLL [Seq. ID No. 12]), was compared to other peptides using an RMA-S stabilization assay as previously described (16) and turned out to be an excellent K^b binder, comparable to SSI. The results of this comparison are summarized in Fig. 5 (SSI (open diamonds, positive control), a D^d-binding peptide, HIV-10 (RGPGRAFVTI [Seq. ID No. 21], filled squares, negative control), TWH (filled circles) and its heteroclitic variant, TAY (filled triangles) at indicated concentrations). In this type of assay, % maximal stabilization provides a direct correlate of peptide binding (16). The experiment for which data is presented is representative of three such assays.

Next, we immunized B6 mice with the TAY and TWH peptides using both pep/TM and genetic methods. Fig. 6A shows CTL activity of anti-TAY CTLs against K^b expressing target cells pulsed with 1 μM TAY (closed squares) or TWH (open squares). TAY-induced CTLs lyse K^b-expressing target cells pulsed with TWH. Lysis of control target cells (<10% at any point) was subtracted from the shown values. Target cells were pulsed with peptides and a chromium release assay performed as described (19). Cumulative results from several experiments of this type are shown in Table 1. As shown in Fig. 6B, TWH is naturally processed in vivo, and can serve as a target for anti-TAY CTLs. The gp75-positive B16 melanoma line, but not its gp75 negative variant (B78.H1) is efficiently lysed by anti-TAY CTLs. B16 (filled squares), B78.H1 (open circles), or B78H.1 pulsed with 10μM of the TWH peptide (open squares), were used as targets in a standard chromium release assay, after a 24 hour incubation with 10 U/ml of IFNγ to induce MHC class I expression. The extent of class I induction was confirmed by flow cytometry, and was similar for both tumor lines (not shown). Similar results were obtained with the ex vivo explanted B16 melanoma (these cells express high levels of MHC class I molecules owing to class I upregulation in vivo). Results

are shown for three independent CTL lines, each one depicted by a different type of line, and are representative of six lines tested so far.

For both immunization protocols, successful CTL priming was obtained only with the engineered, and not the native peptide (Table 1). Importantly, the anti-TAY CTLs lysed target cells pulsed with TWH in vitro, revealing that TAY exhibits the heteroclitic properties for TWH (Fig. 6A). The well characterized melanoma, B16 (29), and its radiation-induced gp75 loss mutant, B78H.1 (29), were next used as *in vitro* targets for the anti-TAY CTL lines (Fig 6B). Despite comparable surface expression of K^b after induction with interferon-γ, B16 (closed squares), but not B78H.1 (open circles), was efficiently lysed (Fig. 6B). Importantly, TWH-peptide sensitized B78H.1 cells were also efficiently lysed, showing that such cells expressed sufficient levels of MHC class I molecules for CTL lysis. These results show that: (I) peptide priming was antigen-specific; (ii) gp75 TWH peptide was naturally processed in the class I pathway; and (iii) the TWH:K^b complexes were expressed at high enough levels on the melanoma cell surface to serve as targets for CTL attack.

We next investigated the potential of TAY as a heteroclitic gp75 vaccine to protect against tumor challenge. Ten mice per group were vaccinated with TAY/TM or TWH/TM, as described (19). Seven days later, they were challenged with 10^5 B16 melanoma cells per mouse, s.c. in the flank. Tumor measurements, number of experiments and result presentation was as in Figures 3A-3G and 4A-4C. Typically, the tumors became palpable after 15 days. Numbers represent the ratio of tumor-free mice to total mice challenged in each group over a period of at least 60 days. The result are summarized in Figs. 7A and 7B and Table, which show tumor growth and incidence in mice vaccinated with TWH or TAY and challenged with B16. Of the mice that were vaccinated with the heteroclitic vaccine, TAY, 100% were protected in that experiment (Figs. 7A and 7B), and 90% were protected in two experiments (Table 2). By contrast, minimal (if any) protection was conferred upon the mice vaccinated with the wild type peptide TWH (Figs. 7A and 7B and Table 2). In day 3 tumors, the same priming regimen did not result in tumor eradication (Table 2), possibly owing to differences in tumor biology between the melanoma and a lymphoma, differences in inherent immunogenicity of the tumors and other factors. Together with the results in the lymphoma model and previous studies *in vitro* (30-32), the above results establish the principles and

establish the applicability of rationally designed heteroclitic vaccination to tumor immunity *in vivo*.

These results demonstrate the applicability and the *in vivo* efficacy of heteroclitic CTL vaccines. The approach to substitute the buried, MHC-contacting residues of a poorly binding peptide, but leave the solvent-exposed TCR-contacting residues intact, was previously used to generate CTLs that killed virally infected (30) or tumor (31,32) targets expressing poorly immunogenic peptides *in vitro*. Furthermore, heteroclitic peptides of the human tyrosinase were shown to be more effective in stimulating $CD4^+$ cells *in vitro* (31). Most recently, in a heterologous vaccination model, a fortuitous presence of a peptide with heteroclitic properties primed T cells that exhibited antitumor activity upon *in vivo* adoptive transfer (33). Using rational epitope identification and engineering and *in vivo* tumor challenge, we now demonstrate the *in vivo* potency of this strategy. The amino acid sequences of tumor antigens can be easily examined to select candidate MHC-binding peptides that can target the tumor for CTL attack. The increasing wealth of the available structural data about MHC:peptide and MHC:peptide:TCR interactions (10-12, 27) can then be used to rationally design candidates for heteroclitic vaccines. Once the best candidates are identified, they can be used individually or in a cocktail vaccine (19, R.Dyall and L Weber, unpublished data). The latter would maximize the odds of success and minimize the risk of tumor escape by epitope mutation. Of course, similar principles can be applied equally well against intracellular pathogens.

Our data also touch upon the issue of self tolerance and tumor immunotherapy. As recently discussed (8), it is now clear that many self differentiation antigens do not induce complete tolerance through deletion of self-reactive lymphocytes from the immune repertoire. In that regard, an important advantage of poor MHC binders derived from self proteins may be that they are unlikely to tolerize T cells (as the signal 1 they provide is not strong enough), sparing a precursor CTL population that can be activated by an appropriate heteroclitic vaccine. Our results with the gp75 peptide TWH lend experimental support to this view, suggesting that this class of weak differentiation antigens could become a potential targets for tumor therapy. Of interest, in the experiments described here, we did not notice any overt signs of autoimmunity (including depigmentation), that one might expect if a melanocyte antigen, such as gp75, is used to target the tumor for lysis by heteroclitic CTLs. Indeed, such

autoimmune manifestations frequently accompany productive anti-melanoma immunity in mice and have been suggested in humans (26 and references therein). At present, it is unclear whether CTLs induced by heteroclitic vaccination are less prone to induce autoimmunity, or whether the results observed were due to the particular peptide chosen. This issue is currently being addressed experimentally.

REFERENCES

1. Houghton, A.N. 1994. Cancer antigens: immune recognition of self and altered self. J. Exp. Med. 180:1-4.

2. Bretscher, P., and M. Cohn. 1970. A theory of self-nonself discrimination. Science 169:1042-1049.

3. Jenkins, M.K., J.D. Ashwell, and R.H. Schwartz. 1988. Allogeneic non-T spleen cells restore the responsiveness of normal T cell clones stimulated with antigen and chemically modified antigen-presenting cells. J. Immunol. 140:3324-3330.

4. Schwartz, R.H. 1990. A cell culture model for T lymphocyte clonal anergy. Science 248:1349-1356.

5. Nossal, G.V.J. 1994. Negative selection of lymphocytes. Cell 76:229-240.

6. Vukmanović, S., M.J. Bevan, and K.A. Hogquist. 1993. The specificity of positive selection: MHC and peptides. Immunol. Rev. 135:51-66.

7. Sette, A., A. Vitiello, B. Reherman, P. Fowler, R. Nayersina, W.M. Kast, C.J.M. Melief, C. Oseroff, L. Yuan, J. Ruppert, J. Sidney, M.F. del Guercio, S. Southwood, R.T. Kubo, R.W. Chesnut, H.M. Grey, and F.V. Chisari. 1994. The relationship between class I binding affinity and immunogenicity of potential cytotoxic T cell epitopes. J. Immunol. 153:5586-5597.

8. Robbins P.F., and Y. Kawakami. 1996. Human tumor antigens recognized by T cells. Curr. Opinion Immunol. 8:628-636.

9. Solinger, A.M, M.E. Ultee, E. Margoliash, and R.H. Schwartz. 1979. T-lymphocyte response to cytochrome c. Demonstration of a T-cell heteroclitic proliferative response and identification of a topographic antigenic determinant on pigeon cytochrome c whose immune recognition requires two complementing major histocompatibility complex-linked immune response genes. J. Exp. Med. 150:830-848

10. Madden, D.R. 1995. The three dimensional structure of peptide-MHC complexes. Annu. Rev. Immunol. 13:587-622.

11. Garcia, C.G., M. Degano, R.L. Stanfield, A. Brunmark, M.R. Jackson, P.A. Peterson, L. Teyton, and I.A. Wilson. 1996. An αβ T cell receptor structure at 2.5 A and its orientation in the TCR-MHC complex. Science 274:209-216.

12. Garboczi, D.N., P. Ghosh, U. Utz, Q. R. Fan, W.E. Biddison, and D.C. Wiley. 1996. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. Nature. 384:134-141.

13. Boehncke, W.H., T. Takeshita, C.D. Pendleton, R.C. Houghten, S. Sadegh-Nasseri, L. Racioppi, J.A. Berzofsky, and R.N. Germain. 1993. The importance of dominant negative effects of amino acid side chain substitution in peptide-MHC molecule interactions and T cell recognition. J. Immunol. 150: 331-339.

14. England, R.D., M.C. Killberg, J.L. Conette, and J.A. Berzofsky. 1995. Molecular analysis of a heteroclitic T cell response to the immunodominant epitope of sperm whale myoglobin. Implications for peptide partial agonists. J. Immunol. 155:4295-4306.

15. van der Burg, S.H., M.J. Visseren, R.M. Brandt, W.M. Kast, and C.J. Melief. 1996. Immunogenicity of peptides bound to MHC class I molecules depends on the MHC-peptide complex stability. J. Immunol. 156:3308-3314.

16. Dyall, R., D.H. Fremont, S.C. Jameson and J. Nikolić-Žugić. 1996. T cell receptor (TCR) recognition of MHC Class I variants: Intermolecular second-site reversion provides evidence for peptide/MHC conformational variation. J. Exp. Med. 184:253-258.

17. Yewdell, J., C. Lapham, I. Bacik, T. Spies, and J. Bennink.1994. MHC-encoded proteasome subunits LMP2 and LMP7 are not required for efficient antigen presentation. J. Immunol. 152:1163-1170.

18. Anderson, K., P. Cresswell, M. Gammon, J. Hermes, A. Williamson, and H. Zweerink. 1991. Endogenously synthesized peptide with an endoplasmic reticulum signal sequence sensitizes antigen processing mutant cells to class I-restricted cell-mediated lysis. J. Exp. Med. 174:489-492

19. Dyall, R., L.V. Vasović, A. Molano, and J. Nikolić-Žugić. 1995. CD4-independent priming of murine CTLs by optimal MHC class I-restricted peptides in vivo. Int. Immunol. 7:1205-1212.

20. Ross, H., L.W. Weber, S. Wang, Y. Takechi, G. Piskun, R. Dyall, J. Nikolić-Žugić, A.N. Houghton, and J.J. Lewis. 1997. Priming for T cell mediated rejection of tumors by cutaneous DNA immunization. Clin. Cancer Res. 3:2191-2196.

21. Ljunggren, H.G., and K. Karre. 1985. Host resistance directed selectively against H-2 deficient lymphoma variants. Analysis of the mechanism. J. Exp. Med. 162:1745-1759.

22. Vijayasaradhi, S., B. Bouchard, and A.N. Houghton. 1990. The melanoma antigen gp75 is the human homologue of the mouse *b* (*Brown*) locus gene product. J. Exp. Med. 171:1375-1380.

23. Houghton, A.N., S. Vijayasaradhi, B. Bouchard, C. Naftzger, I. Hara, and P.B. Chapman. 1993. Recognition of autoantigens by patients with melanoma. Ann.NY Acad.Sci. 690:59-68.

24. Wang R.F., E. Appella, Y. Kawakami, X. Kang, and S.A. Rosenberg. 1996. Identification of TRP2 as a human tumor antigen recognized by cytotoxic T lymphocytes. J. Exp. Med. 184:2207-2216.

25. Hara, I, Y. Takechi, and A.N. Houghton. 1995. Implicating a role for immune recognition of self in tumor rejection: passive immunization against the BROWN locus protein. J. Exp. Med. 182:1609-1614.

26. Naftzger, C., Y. Takechi, H. Kohda, I. Hara, S. Vijayasaradhi, and A.N. Houghton. 1996. Immune response to a differentiation antigen induced by altered antigen: a study of tumor rejection and autoimmunity. Proc. Natl. Acad. Sci. USA 93:14809-14814.

27. Rammensee, H-G., K. Falk, and O. Rotzschke. 1993. Peptides naturally presented by MHC class I molecules. Annu. Rev. Immunol. 11:213-244.

28. Shibahara,S., Y. Tomita, T. Sakakura, C. Nager, B. Chaudhuri, and R. Muller.1986. Cloning and expression of cDNA encoding mouse tyrosinase. Nucleic Acids Res. 14:2413-2427.

29. Takechi, Y., I. Hara, C. Naftzger, Y. Xu, and A.N. Houghton. 1996. A melanosomal protein is a cell surface target for melanoma therapy. Clin. Cancer Res. 2:1837-1842.

30. Lipford, G.B., S. Bauer, H. Wagner and K. Heeg. 1995. Peptide engineering allows cytotoxic T-cell vaccination against human papilloma virus tumor antigen, E6. Immunology 84:298-303.

31. Topalian, S.L., M.I. Gonzales, M. Parkhurst, Y.F. Li, S. Southwood, A. Sette, S.A. Rosenberg and P.F. Robbins. 1996. Melanoma-specific $CD4^+$ T cells recognize nonmutated HLA-DR-restricted tyrosinase epitopes. J. Exp. Med. 183:1965-1971.

32. Bakker, A.B.H., S.H. van der Burg, R.J. Huijbens, J-W. Drijfhout, C.J.M. Melief, G.J. Adema and C.G. Figdor. 1997. Analogues of CTL epitopes with improved MHC class-I binding capacity elicit anti-melanoma CTL recognizing the wild-type tumor epitope. Int. J. Cancer 70:302-309.

33. Overwijk, W.W., A. Tsung, K.R. Irvine, M.R. Parkhurst, T.J. Goletz, K. Tsung, M.W. Carroll, C. Liu, B. Moss, S.A. Rosenberg and N.P. Restifo. 1998. gp 100/pmel 17 is a murine tumor rejection antigen: Induction of "self:-reactive tumoricidal T cells using high-affinity, altered peptide ligand.

We claim:

1. A method for inducing a cellular immune response to a non-immunogenic or weakly immunogenic target peptide expressed by tumor cells of a mammalian subject, comprising administering to the mammalian subject an amount of a therapeutic antigen effective to induce a cellular immune response to the target peptide, wherein the therapeutic antigen comprises an immunogenic portion having an MHC-binding domain which binds to the major histocompatability complex (MHC) and an immune-recognition domain which is recognized by T-cells, and wherein the therapeutic antigen is derived from the target peptide such that the MHC-binding portion binds to MHC with a greater affinity than the target peptide without material alteration of the immune-recognition portion.

2. The method of claim 1, wherein the target peptide and the immunogenic portion of the therapeutic antigen each consist of from 8 to 14 amino acids.

3. The method of claim 1, wherein the therapeutic antigen further comprises a sorting signal for directing trafficking of the therapeutic antigen to the endoplasmic reticulum.

4. The method of claim 2, wherein the target peptide and the immunogenic portion of the therapeutic antigen each consist of from 8 to 14 amino acids.

5. The method of claim 1, wherein the therapeutic antigen is administered by administration of a nucleic acid encoding the therapeutic antigen, which nucleic acid is expressed in the mammalian subject.

6. The method of claim 5, wherein the target peptide and the immunogenic portion of the therapeutic antigen each consist of from 8 to 14 amino acids.

7. The method of claim 1, wherein the therapeutic antigen further comprises a sorting signal for directing trafficking of the therapeutic antigen to the endoplasmic reticulum or endosomes.

8. The method of claim 7, wherein the target peptide and the immunogenic portion of the therapeutic antigen each consist of from 8 to 14 amino acids.

9. The method of claim 1, wherein the MHC-binding domain binds to an MHC Class I molecule and the immune-recognition domain binds to a cytotoxic T cell.

10. The method of claim 1, wherein the MHC-binding domain binds to an MHC Class II molecule and the immune-recognition domain binds to a CD4+ T cell.

11. The method of any of claims 1 to 8, wherein the target peptide binds to HLA-A* 0201.

12. The method of any of claims 1 to 10, wherein the target peptide is a self-peptide expressed in normal and tumor tissues of the mammalian subject.

13. The method of claim 12, wherein the target peptide derived from is gp75.

14. The method according to claim 13, wherein the therapeutic antigen has the sequence TAYRYHLL (Seq. ID No. 12).

15. The method of claim 12, wherein the target peptide is selected from the group consisting of telomerase reverse transcriptase peptide, CD20 peptides and Prostate PSMA peptide.

16. The method of any of claims 1 to 10, wherein the target peptide is a Herpes simplex glycoprotein B peptide and the therapeutic antigen is SSIEFARL (Seq. ID No. 10).

17. A method for preparation of a vaccine against a non-immunogenic or weakly immunogenic target protein expressed by tumor cells of a mammalian subject, comprising the steps of:

(a) identifying an 8-14 amino acid target peptide within the target protein, said target peptide including an MHC-binding domain which binds with low affinity to the major histocompatability complex (MHC) and an immune-recognition domain which is recognizable by T-cells;

(b) preparing one or more test peptides in which the MHC binding domain is modified and testing the test peptide for binding affinity to the MHC;

(c) selecting a test peptide from among the one or more test peptides for use in the vaccine, said selected peptide having a greater binding affinity than the target peptide; and

(d) preparing a vaccine composition comprising the selected peptide or a polynucleotide encoding the selected peptide in a pharmaceutically acceptable carrier.

18. The method of claim 17, wherein the target peptide binds to HLA-A* 0201.

19. The method of claim 17, further comprising the step of coupling the selected peptide with a sorting signal for directing trafficking of the therapeutic antigen to the endoplasmic reticulum or endosomes.

20. The method of claim 17, wherein the MHC-binding domain binds to an MHC Class I molecule and the immune-recognition domain binds to a cytotoxic T cell.

21. The method of claim 17, wherein the MHC-binding domain binds to an MHC Class II molecule and the immune-recognition domain binds to a CD4+ T cell.

22. The method of any of claims 17-21, wherein the target protein is a self-protein expressed in normal and tumor tissues of the mammalian subject.

23. The method of any of claims 17-21, wherein the target peptide derived from is gp75.

24. The method of any of claims 17-21, wherein the target peptide is selected from the group consisting of telomerase reverse transcriptase peptide, CD20 peptides and Prostate PSMA peptide.

25. The method of any of claims 17-21, wherein the therapeutic antigen has the sequence TAYRYHLL (Seq. ID No. 12).

26. The method of any of claims 17-21, wherein the target peptide is a Herpes simplex glycoprotein B peptide and the therapeutic antigen is SSIEFARL (Seq. ID No. 10).

27. A therapeutic immunogen comprising an MHC-binding domain which binds to the major histocompatability complex (MHC) of a mammalian subject and an immune-recognition domain which is recognized by T-cells. wherein the therapeutic immunogen is derived from a non-immunogenic or weakly immunogenic target peptide expressed by tumor cells of the mammalian subject such that the MHC-binding portion binds to MHC with a greater affinity than the target peptide without material alteration of the immune-recognition portion.

28. The therapeutic immunogen of claim 27, further comprising a sorting signal for directing trafficking of the therapeutic antigen to the endoplasmic reticulum or endosomes.

29. The therapeutic immunogen of claim 27, wherein the MHC-binding domain binds to an MHC Class I molecule and the immune-recognition domain binds to a cytotoxic T cell.

30. The therapeutic immunogen of claim 27, wherein the MHC-binding domain binds to an MHC Class II molecule and the immune-recognition domain binds to a CD4+ T cell.

31. A polynucleotide expressible in a mammalian subject, comprising a series of bases encoding the therapeutic immunogen of any of claims claim 27 to 30.

32. A vaccine composition for treatment or prevention of a tumor in a mammalian subject, comprising a therapeutic immunogen in accordance with any of claims claim 27 to 30, or a polynucleotide expressible in the mammalian subject comprising a series of bases encoding the therapeutic immunogen of any of claims claim 27 to 30, together with a pharmaceutically acceptable carrier.

33. The composition according to claim 32, wherein the pharmaceutically acceptable carrier comprises an adjuvant.

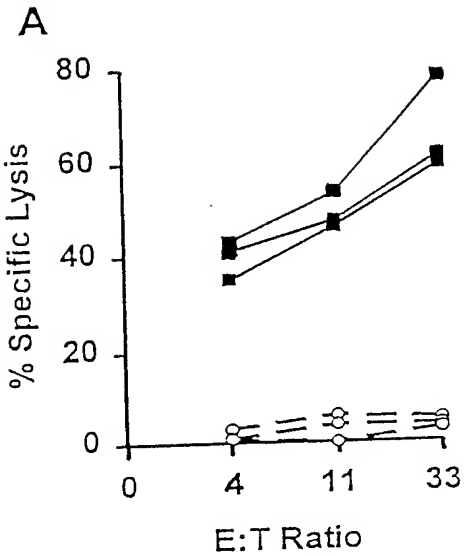

A
80
60
40
20
0
% Specific Lysis
0
4
11
33
E:T Ratio

FIG. 1A

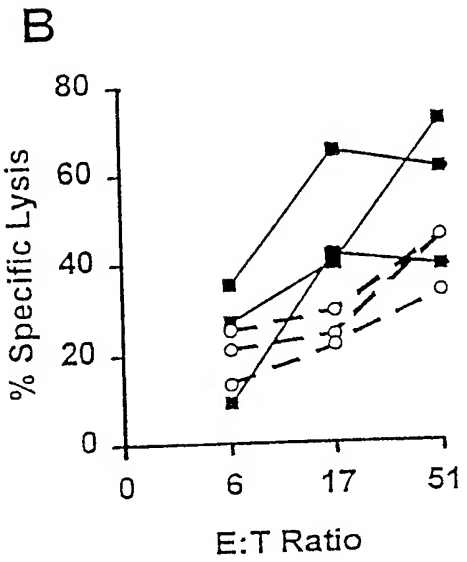

B
80
60
40
20
0
% Specific Lysis
0
6
17
51
E:T Ratio

FIG. 1B

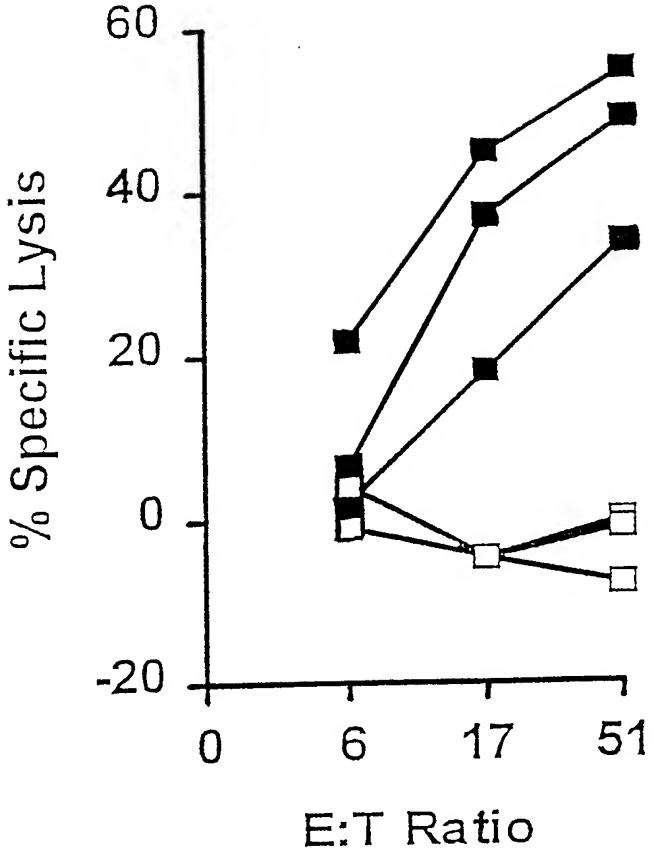


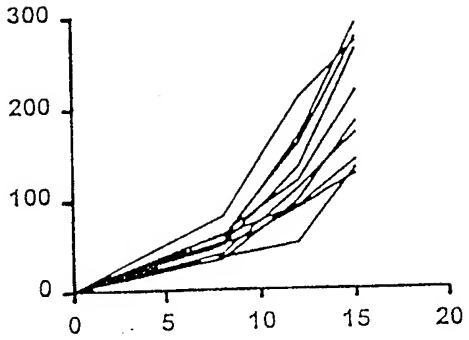
FIG. 2

FIG. 3A FIG. 3B FIG. 3C FIG. 3D

FIG. 3E FIG. 3F FIG. 3G

pCDNA3 Tumor free

FIG. 4A

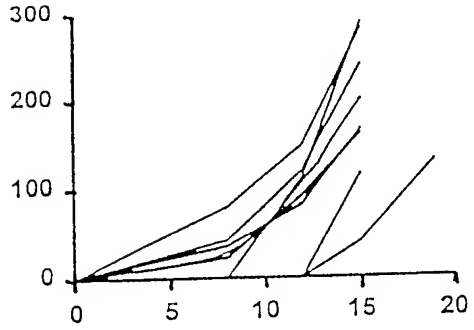


0/10

pERIS-SEI

Tumor size in mm^2

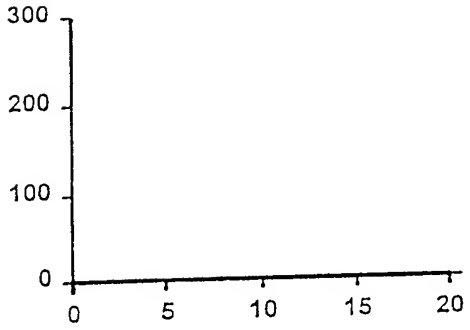
FIG. 4B



0/10

pERIS-SSI

FIG. 4C



9/10

Days post-challenge

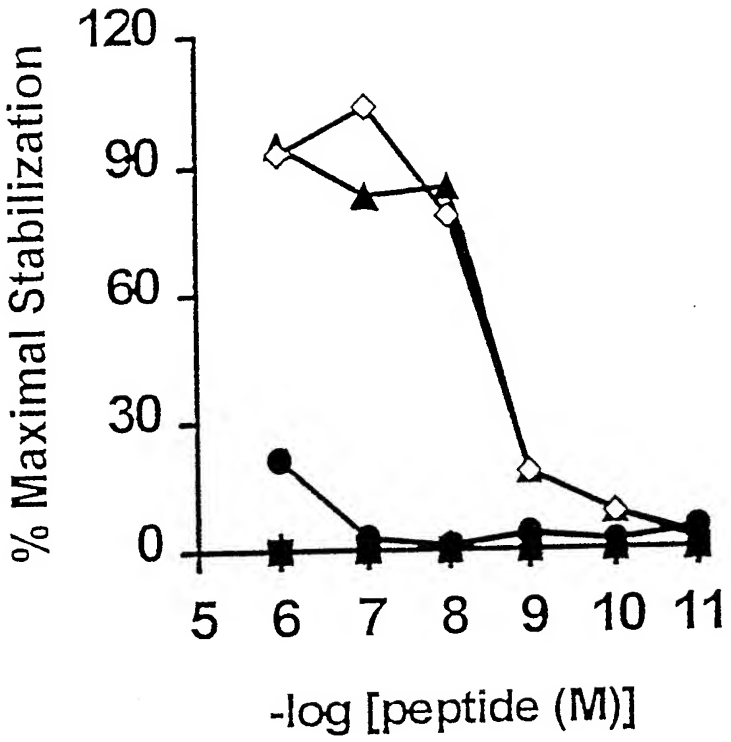

% Maximal Stabilization
120
90
60
30
0
5
6
7
8
9
10
11
-log [peptide (M)]

FIG. 5

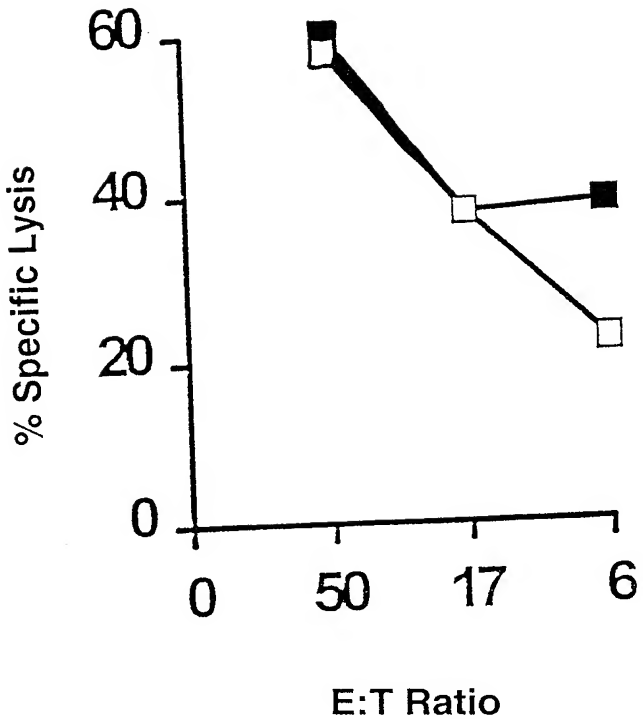


FIG. 6A

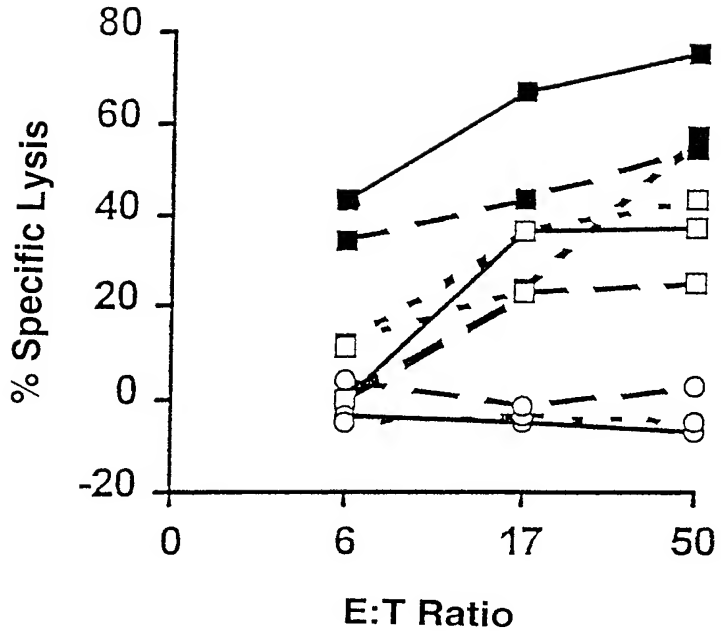


FIG. 6B

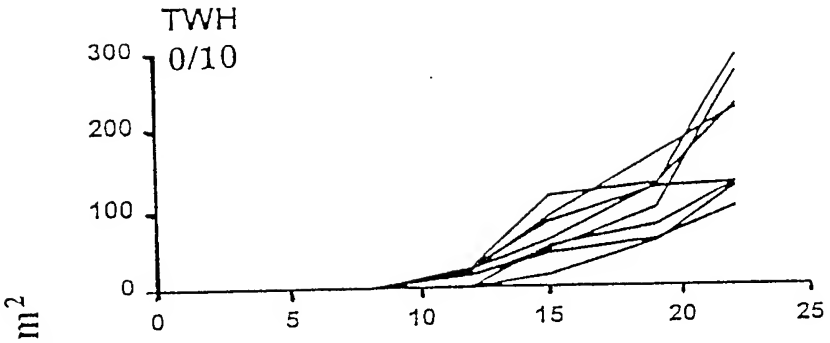
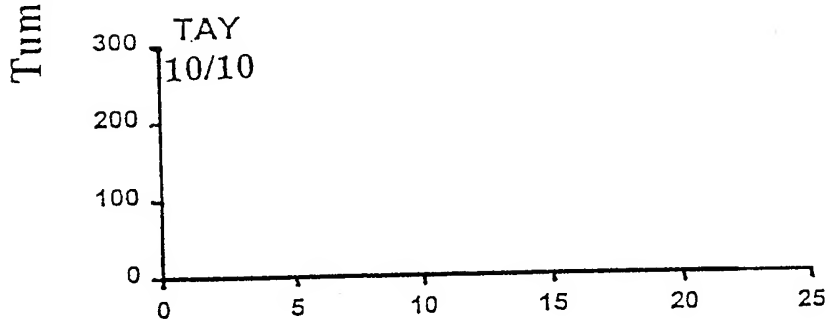


FIG. 7A



Tumor size mm^2

Days post-challenge

FIG. 7B

SEQUENCE LISTING

<110> Nikolic-Zugic, Janko
Dyall, Ruben
Houghton, Alan N.

<120> Vaccination Strategy to Prevent and Treat Cancers

<130> MSK.P-042-WO

<140>
<141>

<150> 60/106,339
<151> 1998-10-30

<150> 60/089,055
<151> 1998-06-12

<160> 23

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<212> PRT
<213> Vesicular stomatitis virus

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Ala

<210> 3
<211> 8
<212> PRT
<213> Chicken

<220>
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1 5

<210> 4
<211> 9
<212> PRT
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<220>
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<220>
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1 5

<210> 10
<211> 8
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1 5

<210> 11
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<212> PRT
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<220>
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Thr Trp His Arg Tyr His Leu Leu
1 5

<210> 12
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<223> Description of Artificial Sequence: amplification primer

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<212> DNA
<213> Artificial Sequence

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<223> Description of Artificial Sequence: amplification primer

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<210> 15
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<212> DNA
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<220>
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<213> Artificial Sequence

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<213> Artificial Sequence

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<223> Description of Artificial Sequence: amplification primer

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<210> 20
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: amplification primer

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<211> 10
<212> PRT
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<223> HIV-10 peptide

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<210> 22
<211> 17
<212> PRT
<213> HUMAN

<220>
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Gly

<210> 23
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<212> PRT
<213> HUMAN

<220>
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1 5